SYNERGISTIC EFFECTS OF MEDIATED GENE THERAPY WITH GEN2 (HSV-TK) AND GEN1018 (IL-12) IN AN EXPERIMENTAL MODEL OF COLORECTAL CANCER

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ABSTRACT

Background: GEN2 is a clinical-stage retrovector that integrates into rapidly dividing cancer cells and delivers an improved gain-of-function variant of herpes simplex virus thymidine kinase (HSV-TK) as a prodrug activator ('suicide') gene. Consequently, GEN2 induces cancer cell death upon treatment with a prodrug such as valganciclovir (VGCV). Immune cell activation can be further promoted inside the tumor microenvironment (TME) by immunocytokines, such as Interleukin-12 (IL-12). IL-12 potently activates anti-tumor immune responses through enhancement of natural killer (NK) cell and CD8+ T lymphocyte cytotoxicity, but systemic administration of IL-12 has been reported to be highly toxic in clinical trials. Accordingly, here we evaluated the utility of the GEN2 vector for HSV-TK suicide gene therapy combined with tumor-localized GEN1018 vector for murine IL-12 (mIL-12) immuno-gene therapy in an experimental model of colorectal cancer.

Methods: Colorectal cancer models were established in immunocompetent 8-10 week old BALB/c mice by subcutaneous injection of 1.5 x 10^5 syngeneic CT26 colorectal cancer cells expressing luciferase as a reporter, and injected intratumorally (IT) with GEN1018 (mIL-12) or GEN2 (HSV-TK) for 3 days. In the second week, VGCV was administered by oral gavage once a day for 5 consecutive days. One of the HSV-TK/VGCV-treated groups was given additional GEN1018 (mIL-12) injections IT for an additional 3 days after completion of the VGCV treatments. All mice were monitored weekly by bioluminescence imaging (BLI) and measurement of tumor size, and tumor tissues were collected for analyses.

Results: Both BLI and tumor size measurements demonstrated that GEN2 (HSV-TK)/VGCV suicide gene therapy in combination with a second administration of GEN1018 (mIL-12) treatment could achieve enhanced long-term tumor growth inhibition, as compared to a single cycle of 3 injections of either GEN2 (HSV-TK)/VGCV or GEN1018 (mIL-12) individually. Tumor growth inhibition in the combined treatment group receiving GEN2 (HSV-TK)/VGCV and 2 cycles of GEN1018 (mIL-12) was associated with significantly prolonged survival compared to all other treatment groups. Immunohistochemistry of tumor samples showed increased CXCL10 expression in the tumor microenvironment, associated with increased recruitment of tumor-infiltrating T cells, in groups treated with multiple doses of GEN1018 (mIL-12).

Conclusions: Direct IT injections of GEN2 (HSV-TK) in combination with GEN1018 (mIL-12) were associated with immunomodulatory effects within the tumor microenvironment, tumor growth inhibition, and improved survival in a model of highly aggressive colorectal cancer.

INTRODUCTION

GEN2 is a clinical stage retrovector, currently being evaluated in a First-in-Human Phase I dose escalation trial for prodrug activator ('suicide') gene therapy in patients with advanced primary or metastatic malignancies in the liver. GEN2 integrates into dividing cancer cells, delivering a gain-of-function variant of the herpes simplex virus thymidine kinase (HSV-TK) suicide gene. The prodrug valganciclovir (VGCV) is converted to ganciclovir which when absorbed by cells transduced with HSV-TK is phosphorylated to ganciclovir triphosphate (1). Cell death is then induced in actively-dividing cancer cells via DNA chain termination, which occurs when DNA polymerases incorporate ganciclovir triphosphate in place of deoxyguanosine triphosphate (2). This induced cell death results in the release of tumor antigens, which can stimulate an immune response and recruit antigen-specific T cells into the TME (3). These activated T cells can result in destruction of neighboring tumor cells, a phenomenon that has been termed as a 'distant' bystander effect (4). However, immunosuppressive mechanisms activated by cancer cells often inhibit anti-tumor immune responses.

GEN1018 is a retrovector encoding interleukin-12 (IL-12), an immunocytokine that can augment anti-tumor immune responses by stimulating the production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) from natural killer (NK) cells and CD8+ cytotoxic T lymphocytes, and promoting their migration, activation, and proliferation following systemic and/or local administration (5). In murine models, mIL-12 can prime and enhance survivability of naïve CD8+ T cells, resulting in improved anti-tumoral activity (6) and making tumors immunologically "hot" (7). However, systemic administration of hIL-12 has led to significant toxicity in clinical trials (8). Here we employed GEN1018 (mIL-12) gene transfer via intratumoral (IT) injection as an approach to elevate local cytokine concentrations and reduce adverse toxicity associated with systemic administration.

In the present study, we evaluated the efficacy of GEN2 vector mediated HSV-TK suicide gene therapy in combination with GEN1018 vector for mIL-12 immuno-gene therapy. Both vectors were delivered intratumorally in a subcutaneous tumor model of syngeneic CT26 murine colorectal cancer in immunocompetent BALB/c mice. In this highly aggressive model, tumor growth vs. pseudoprogression was distinguished by engineering CT26 cells to express the firefly luciferase reporter gene, enabling viability of tumor tissues to be evaluated *in vivo* by bioluminescence imaging (BLI).

METHODS AND MATERIALS

Animals: Eight to ten-week-old immune competent female BALB/c mice were purchased from the Jackson Laboratory and housed at the animal facility at the Children's Hospital Los Angeles (CHLA)/Saban Research Institute (Los Angeles, CA). CHLA Institutional Animal Care and Use Committee (Protocol # 409-19) was approved by the Animal Care Committee of Children's Hospital Los Angeles. The animals were maintained in a sterile environment, and cages, food, and bedding were autoclaved.

CT26-Luc Cell Line: CT26 murine colorectal cancer cells (ATCC) were transduced with a vector expressing the firefly luciferase gene, and selected in Geneticin™ to isolate a stable clonal CT26-Luc cell line that enables bioluminescence imaging to monitor tumor establishment, growth, and metastasis following subcutaneous implantation in syngeneic BALB/c mice. These CT26-Luc cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% Pen-Strep and incubated at 37°C in 5% CO_2.

CT26-Luc Tumor Model: CT26-Luc cells (1.5 x 10^5 cells/150 μL of Phosphate Buffered Saline mixed with 20% Matrigel) were implanted subcutaneously with a 27G needle. Tumor size was measured with a digital caliper, and weekly bioluminescence imaging (BLI) was performed using the IVIS Imaging System (Perkin Elmer). The mice received 200 μL of luciferin (15 mg/mL, Promega) intraperitoneally before *in vivo* imaging.

Vector Injections: Once tumors reached a volume of ~100 mm³, intratumoral injections of GEN2 (HSV-TK) and/or GEN1018 (mIL-12) were initiated. Fifty (50) μL of the vectors were injected homogeneously into 3 or 4 sites of the tumor with a 27G needle once a day for 3 consecutive days.

Valganciclovir (VGCV) Treatment: Once implanted tumors reached 200 mm³ in size, valganciclovir (VGCV [Sigma]) administration was initiated and given orally once a day for 5 consecutive days by oral gavage in unanesthetized mice. VGCV was prepared in ORA-SWEET® (Paddock Laboratories) at a concentration of 2 mg/mL and administered at 5 mL/kg body weight. ORA-SWEET® solution without VGCV was administered to control animals using the same dosage and schedule.

Tumor Tissue Analyses: Animals were euthanized via CO_2 when implanted tumors exceeded 1.5 cm in diameter, or exhibited morbidity, respiratory distress, inability to feed, or dehydration. Following euthanasia, CT26 tumor tissues were collected for analysis. For immunofluorescence (IF) staining, tumor tissues were formalin-fixed, then embedded in paraffin. Five (5) μm tissue sections were deparaffinized and blocked in 5% normal goat serum in Tris-Buffered Saline (TBS), then incubated with FITC conjugated anti-HSV-TK antibody overnight at 4 °C. After washing with TBST (TBS with 0.1% Tween-20), sections were counterstained by DAPI. The slides were visualized under the Olympus BX53 Upright fluorescent microscope (Olympus Corporation).

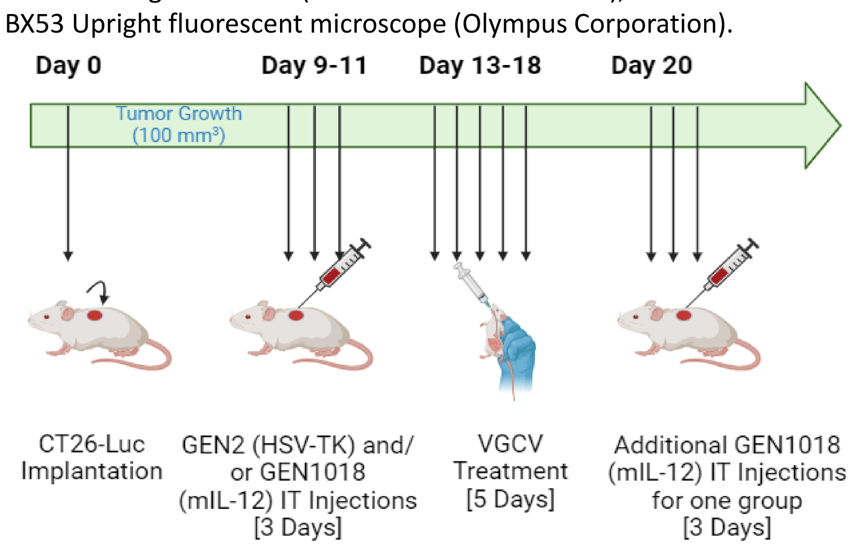


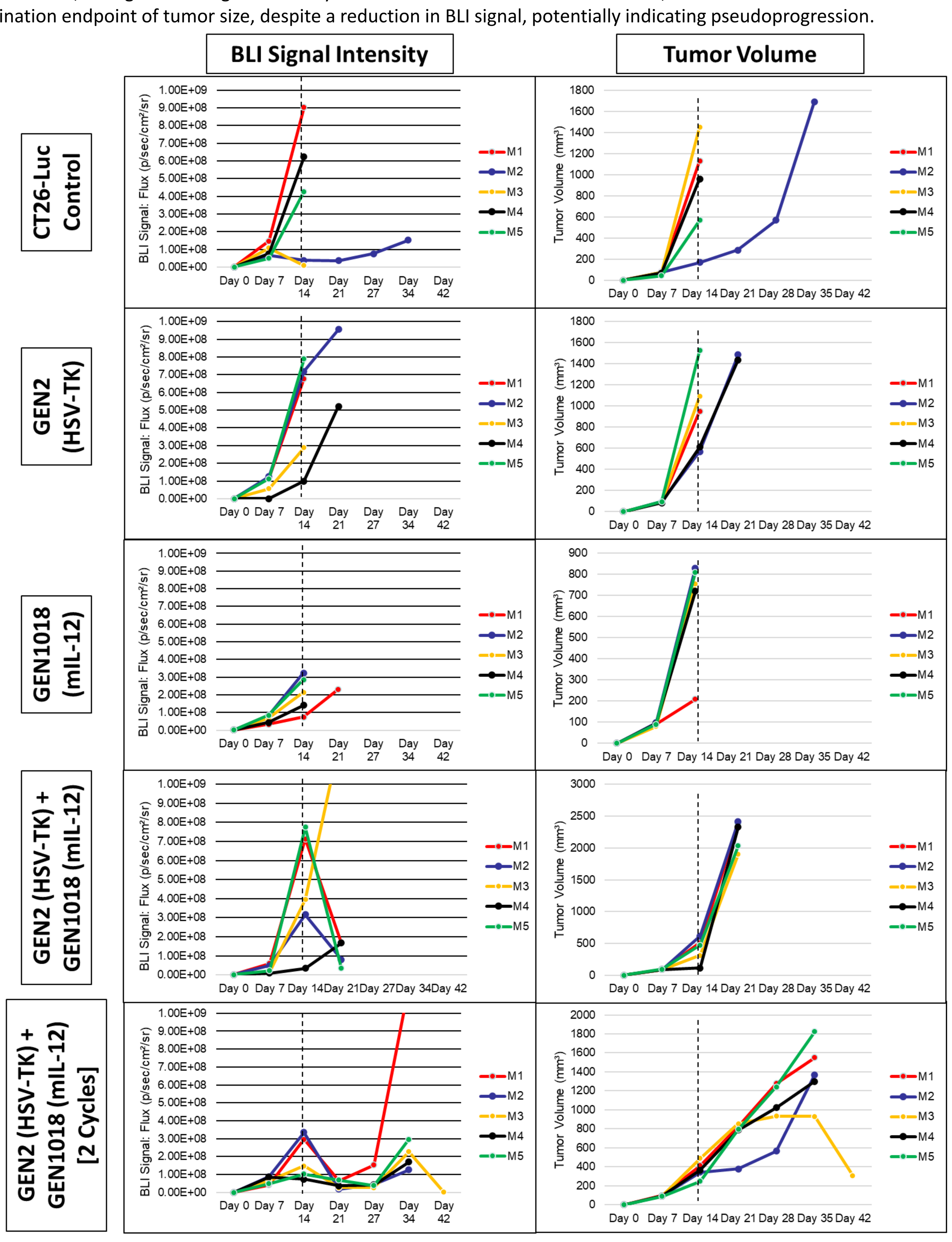
Figure 1: Animal dosing schedule following CT26-Luc implantation. GEN2 (HSV-TK) and/or GEN1018 (mIL-12) IT injections for 3 days were initiated 9 days following CT26-Luc implantation. VGCV treatment for 5 days was initiated 2 days following the last IT injection. One group also received an additional cycle of 3 injections of GEN1018 (mIL-12) 2 days following VGCV treatment. Figure 1 created with BioRender.com.

RESULTS

Figure 2: **Left Panels**: BLI measurements following CT26-Luc implantation in BALB/c mice. Over the 42-day observation period post-CT26-Luc implantation, BLI signals show elevation over time for most of the mice in the CT26-Luc control, GEN2 (HSV-TK), and GEN1018 (mIL-12) groups. However, the combination of GEN2 (HSV-TK) and GEN1018 (mIL-12) reduced the BLI signals in most of the mice, indicating a synergistic effect of the treatments. Dashed line indicates termination timepoint for the majority of control animals.

Right Panels: Tumor volume curves following CT26-Luc implantation in BALB/c mice. Tumor volumes (mm³) were measured with a digital caliper. GEN1018 (mIL-12) with GEN2 (HSV-TK) with VGCV treatment suppressed tumor growth compared to the CT26-Luc control. The additional cycle of GEN1018 (mIL-12) delayed tumor growth until Day 35.

For most of the mice, changes in BLI signal intensity correlate with tumor volume. However, some of the mice were sacrificed based solely on the termination endpoint of tumor size, despite a reduction in BLI signal, potentially indicating pseudoprogression.



RESULTS (CONTINUED)

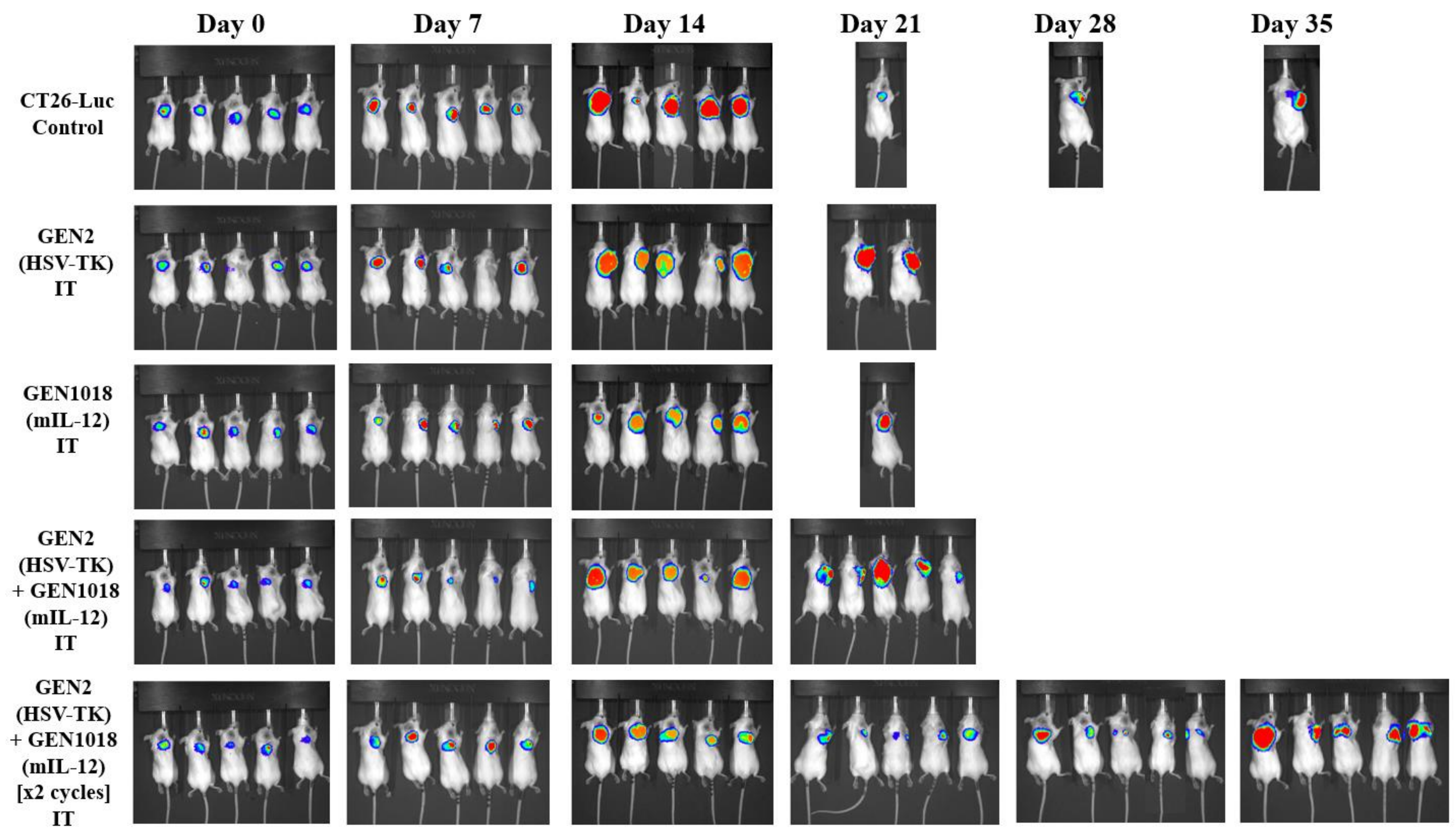


Figure 3: Weekly bioluminescence imaging following CT26-Luc implantation in BALB/c mice. 200 μL luciferin (15 mg/mL) was injected intraperitoneally before *in vivo* imaging using the IVIS Imaging System. GEN2 (HSV-TK) and GEN1018 (mIL-12) with VGCV in combination with a second cycle of GEN1018 (mIL-12) significantly reduced tumor growth compared to the other groups.

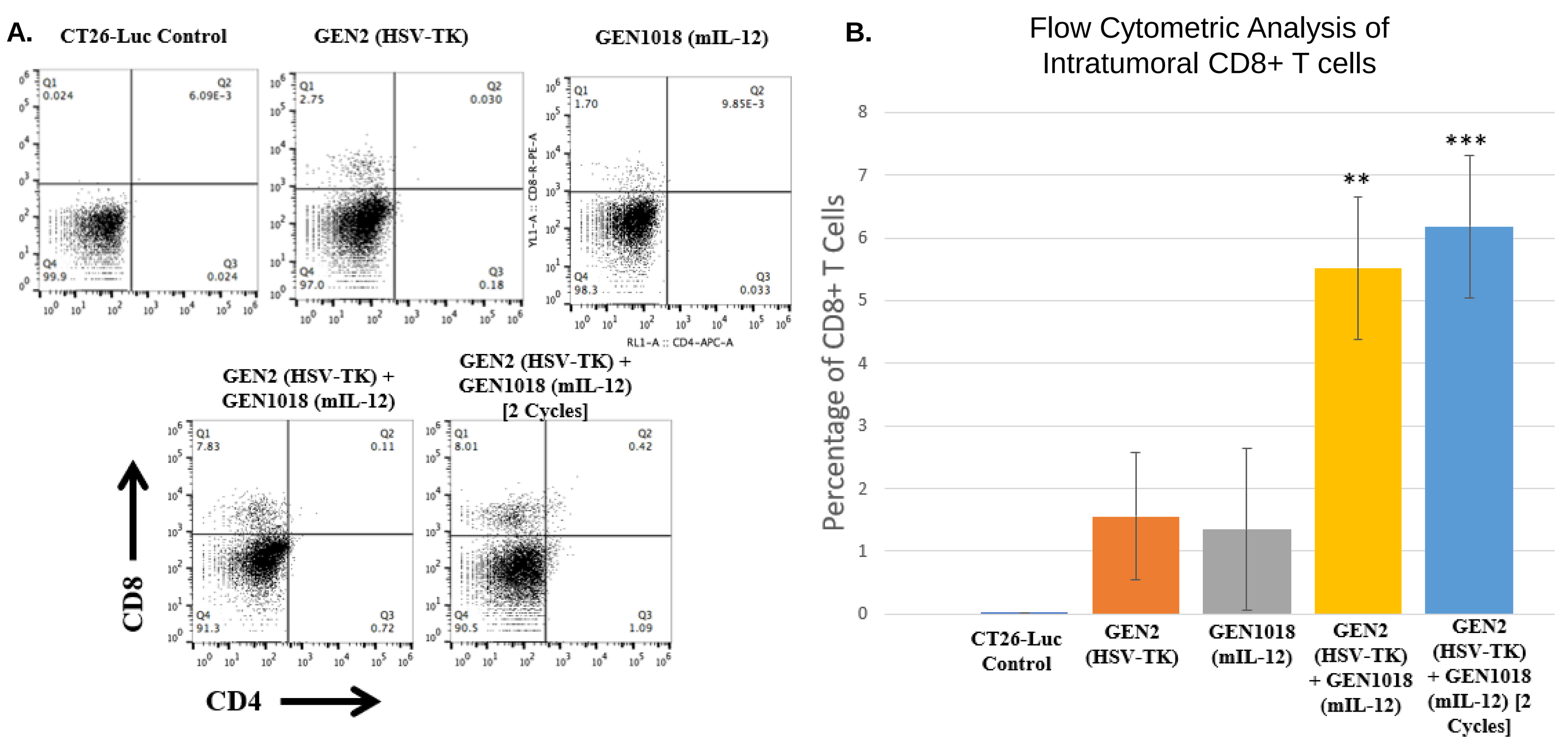


Figure 4: Flow cytometric analysis of tumor samples stained with PE-conjugated anti-CD8 and APC-conjugated anti-CD4 antibodies. (A) Representative data from individual mice in each group. GEN2 (HSV-TK) and GEN1018 (mIL-12) combination treatment groups showed higher levels of CD8+ T cells. (B) Average percentage of intratumoral CD8+ T cells in each treatment group. GEN2 (HSV-TK) and GEN1018 (mIL-12) combination treatment groups showed statistically significant higher percentages of intratumoral CD8+ T cells compared to the CT26-Luc Control group. All data are shown as mean ± SEM (4-5 mice/group). **p<0.01, ***p<0.001.

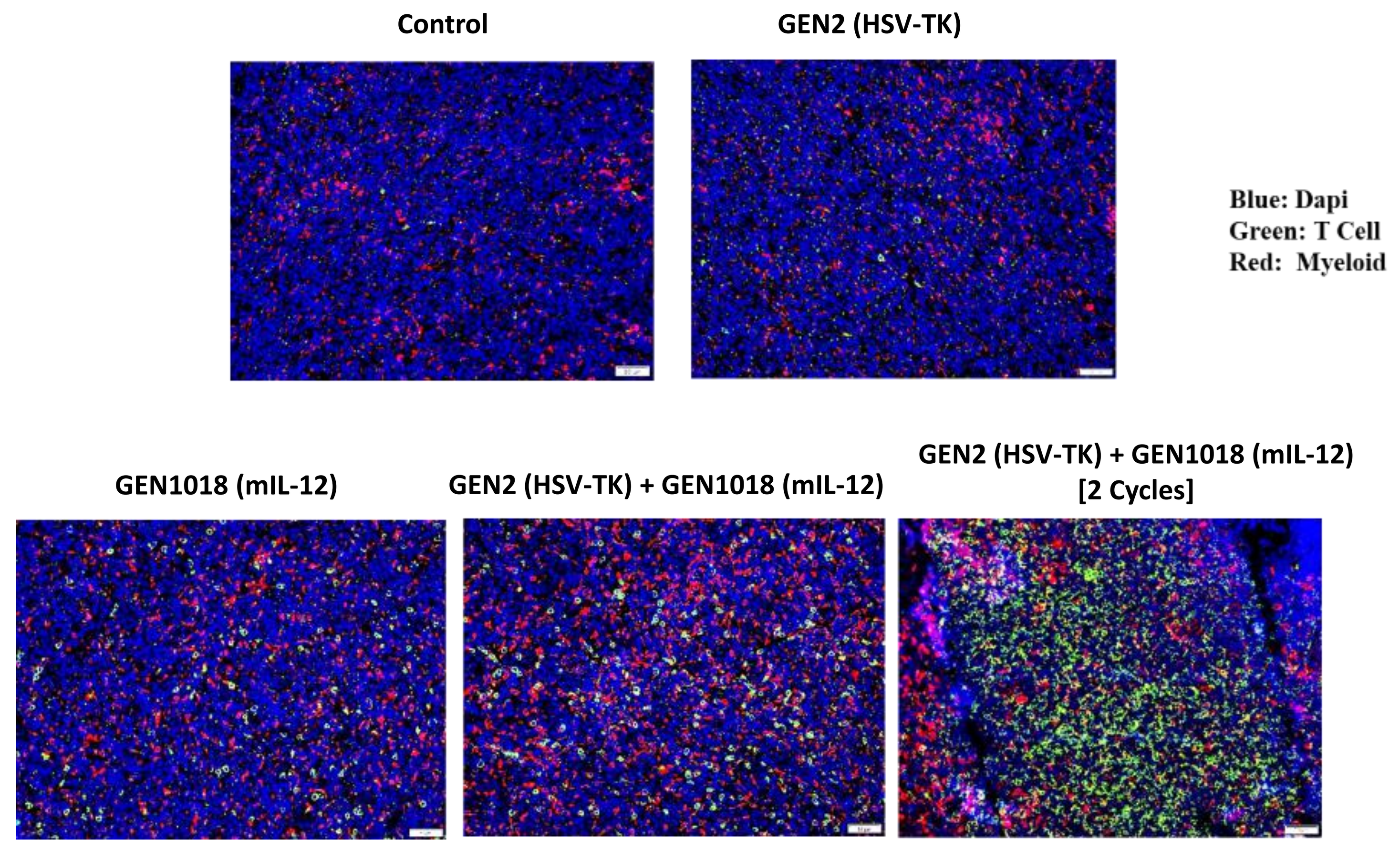


Figure 5: Immunohistochemistry staining of paraffin-embedded tumor tissue with DAPI, CD8, and CD11b. The CT26-Luc control and GEN2 (HSV-TK) had minimal immune cell infiltration. GEN1018 (mIL-12) treatment was associated with an increased number of both myeloid and CD8+ T cells. The additional cycle of GEN1018 (mIL-12) treatment further increased intratumoral infiltration of CD8+ T cells.

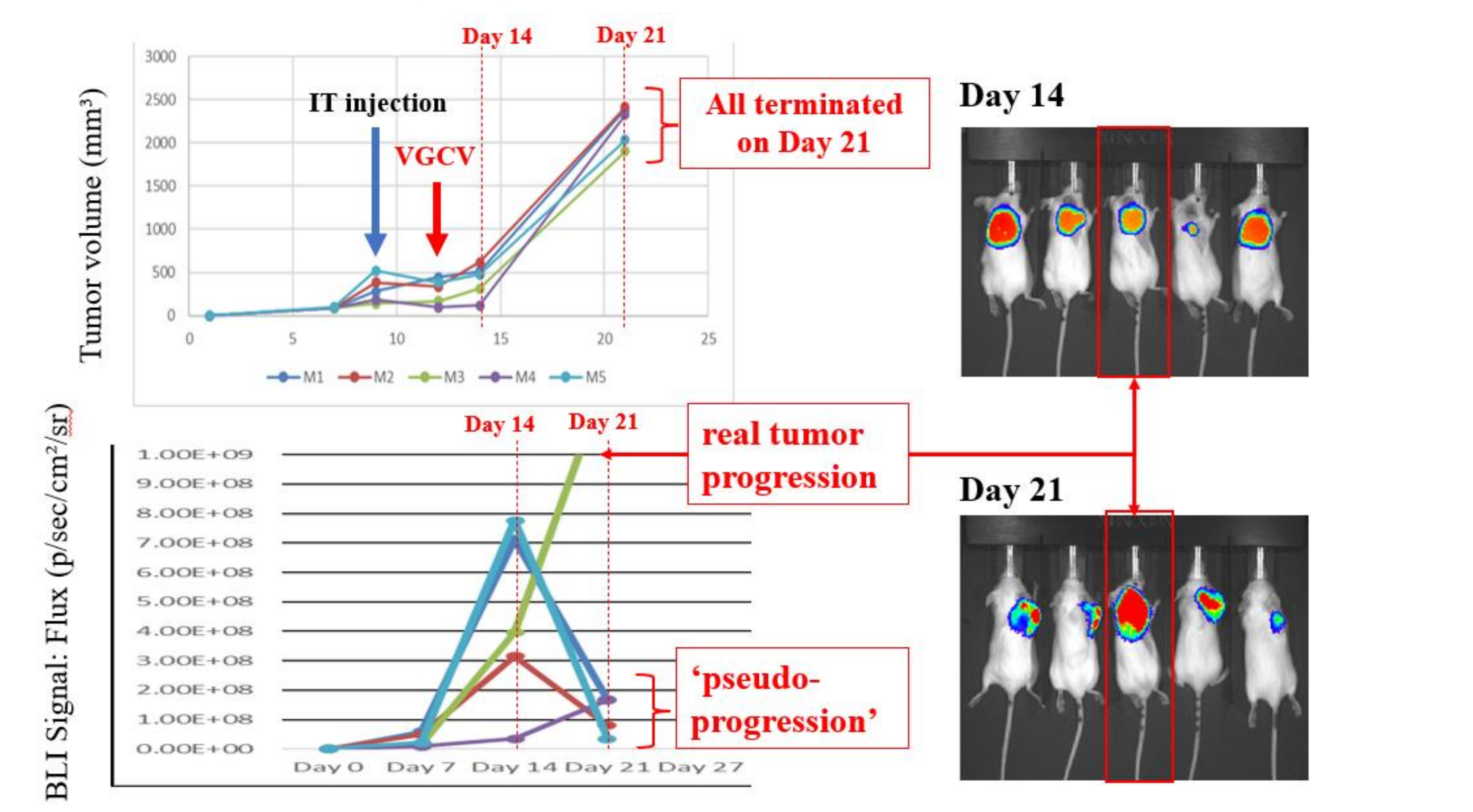


Figure 6: A case for pseudoprogression based on results of individual BALB/c mice after GEN2 (HSV-TK), GEN1018 (mIL-12), and VGCV treatment. Pseudoprogression is a phenomenon when tumor size is increased due to infiltration of immune cells, followed by a delayed decrease in tumor burden (9). Termination criteria were based solely on tumor size; all mice were terminated when implanted tumors exceeded 1.5 cm in diameter. However, some of the mice showed significant decreases in BLI signals despite increased tumor volume, potentially indicating pseudoprogression.

CONCLUSIONS

Here we report that tumor-localized mIL-12 gene therapy in combination with GEN2 suicide gene therapy not only increased recruitment of immune cells into the TME but also suppressed the growth of viable tumor tissue following multiple GEN1018 (mIL-12) injections. Importantly, there was no observable toxicity when mIL-12 encoding vector was administered locally into the tumors.

Direct IT injections of GEN2 (HSV-TK) with VGCV or GEN1018 (mIL-12) individually did not reduce BLI signals or suppress tumor growth over time, but combining GEN2 (HSV-TK) with VGCV and a single cycle of GEN1018 (mIL-12) significantly reduced BLI signals in most of the mice during the 42-day observation period. Notably, tumor volume appeared to continue to increase in some animals despite significantly reduced BLI signals, indicating pseudoprogression. The termination criteria for the mice in this study was based solely on implanted tumors reaching 1.5 cm in diameter. As a result, some animals in the treatment groups were sacrificed prematurely based on tumor size, despite therapeutic efficacy illustrated by a reduction in BLI signal over time. Activation of anti-tumor immune responses was further illustrated by FACS and IHC data, showing increased infiltration of CD8+ T cells in GEN1018 (mIL-12) treated tumors compared to the CT26-Luc control group. The addition of another cycle of GEN1018 (mIL-12) in combination with GEN2 (HSV-TK) and GEN1018 (mIL-12) with VGCV not only had the highest levels of CD8+ T cells but also displayed reduced BLI signals and inhibition of tumor growth compared to the other groups.

These results indicate that combining GEN2 (HSV-TK) suicide gene therapy with GEN1018 (mIL-12) immuno-gene therapy has potential to achieve synergistic effects on inhibiting tumor progression in this highly aggressive colorectal model, and supports further translational and clinical development of this approach.

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GenVivo Inc. is a clinical-stage biotechnology company developing a novel vector platform to deliver mRNA for oncology and infectious diseases.